Effect of Enzyme Immobilization on Thermal Stability

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Research Question:
How does the immobilization of an enzyme (specifically, catalase) affect its tolerance to changes in temperature?

1 Personal Engagement

I decided to study this because I am interested in the development of clean energy technology, and one of the foremost problems that arise when attempting to find innovative uses for enzymes is their chemical tolerance limits for pH and temperature. Efficient manipulation of enzymes could hold the key to major advancements in renewable energy, whether it be the development of a new biofuel or eliminating the need for extreme pressures and temperatures in certain industrial processes. I chose to explore how immobilization affects thermal stability because it is known that immobilization makes enzymes more stable and that high temperature denatures enzymes, but I wanted to see this on a quantitative level rather than just a qualitative level. I selected catalase to study because it is common and easy to access, and the reaction it catalyzes is relatively easy to monitor for the purposes of calculating reaction rates, since oxygen concentration sensors are readily available in most high school lab environments. I chose to work with the Equilibrium Model rather than perform a more basic level of quantitative analysis because I enjoy computational challenges and wanted to get a highly accurate representation of the extent of the thermal stability of catalase in both forms. I would like to delve further into this topic by investigating how the secondary structure of an enzyme can be correlated to temperature tolerance.

2 Background

Catalase (fig.1) is a common enzyme that plays an important role in cellular metabolism by catalyzing the decomposition of hydrogen peroxide, a harmful waste product, into water and oxygen, represented by the reaction $2\,H_2O_2 \longrightarrow 2\,H_2O + O_2$ .Catalase also has various industrial applications, including the preservation of food items, the manufacturing of beverages, and in the breaking down of hydrogen peroxide in wastewater.

An immobilized enzyme is an enzyme attached to an inert, insoluble material such as calcium alginate. Immobilization is a common industrial process and has numerous advantages, one of which is increased resistance to changes in pH and temperature.

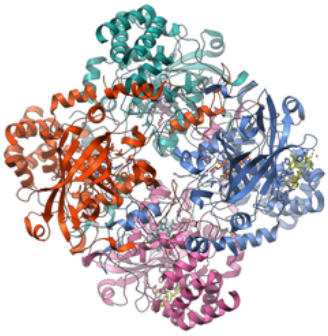

Figure 1: The quaternary structure of catalase.

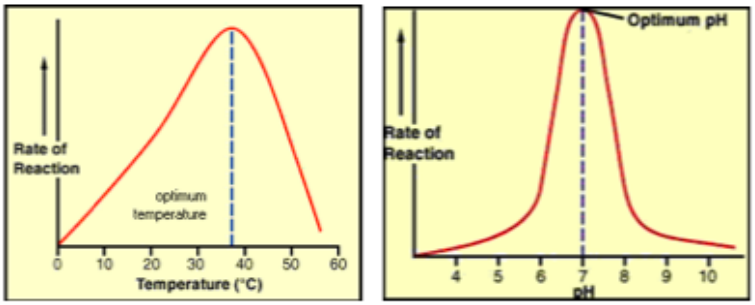

Figure 2: Generalized curves for reaction rate as a function of pH and temperature.

Like all enzymes, catalase in its original and immobilized forms operates most efficiently at an optimal set of conditions. More specifically, changes in temperature and pH can cause the enzyme to denature, or undergo a transformation where molecular structure is compromised in a manner that inhibits proper functionality. As a result, changes in pH and temperature result in enzyme activity curves that are approximately bell-shaped, with local maxima occurring at the optimal pH and temperature values (fig. 2).

If we are interested in determining the temperature tolerance range (thermal stability) of an enzyme, we want to learn more about the shape and spread (standard deviation) of the enzyme activity curve for temperature. The currently accepted model for determining enzyme thermal stability is called the Equilibrium Model [Peterson, 2007], and it provides a complete quantitative description of the variation of enzyme activity with temperature. Using the Equilibrium Model, the variation of enzyme activity with temperature can be expressed by:

$$V_{\max} = \frac{k_{cat} E_0 e^{\frac{-k_{inact} K_{\mathrm{eq}} t}{1+K_{eq}}}}{1 + K_{eq}} \tag{1}$$

Where

$$K_{eq} = e^{\frac{\Delta H_{eq}}{R}} \tag{2}$$

And the variations of k_{cat} (catalytic rate constant) and k_{inact} (rate constant for thermal inactivation) with temperature with temperature are given by:

$$k_{cat} = \frac{k_B T}{h} e^{\frac{-\Delta G^*_{\mathrm{cat}}}{RT}} \tag{3}$$

$$k_{inact} = \frac{k_B T}{h} e^{\frac{-\Delta G^*_{\mathrm{inact}}}{RT}} \tag{4}$$

T_{eq} is the temperature at which the $\frac{E_{\mathrm{act}}}{E_{\mathrm{inact}}}$ equilibrium is at its mid-point ($K_{\mathrm{eq}} = 1$). Under the assumption that $\frac{E_{\mathrm{act}}}{E_{\mathrm{inact}}}$ equilibration is rapid, we can expand equation 1 to:

$$V_{\max} = \frac{k_B T e^{\frac{-\Delta G^*_{\mathrm{cat}}}{RT}} E_0 e[\frac{k_B T e^{\frac{-\Delta G^*_{\mathrm{inact}}}{RT}} e^{(\frac{\Delta H_{\mathrm{eq}}(\frac{1}{T_{\mathrm{eq}}} - \frac{1}{T})}{R})} t]}{h(1+e^{(\frac{\Delta H_{\mathrm{eq}}(\frac{1}{T_{\mathrm{eq}}} - \frac{1}{T})}{R})})}}{h[1 + e^{(\frac{\Delta H_{\mathrm{eq}}(\frac{1}{T_{\mathrm{eq}}} - \frac{1}{T})}{R})}]} \tag{5}$$

And we now have a model which describes the variation of $V_{\max}$ with time and temperature. A glossary of terms is included in table 1.

Variables in the Equilibrium Model, Explained [Daniel, 2010]
E_0: The total concentration of the enzyme in the suspension.
ΔG^*_{cat}: Gibbs' free energy of activation for an enzyme catalyzed reaction, one of the four parameters determined experimentally.
$\Delta G^*_{\text{inact}}$: Gibbs' free energy of activation for the thermal activation of an enzyme, one of the four parameters determined experimentally.
h: Planck's constant, $6.626 * 10^{-23} \frac{J}{K}$
ΔH_{eq}: Change in enthalpy for the transition from the active to inactive form of the enzyme, one of the four parameters determined experimentally. k_B: Boltzmann's constant, $1.381 * 10^{-23} \frac{J}{K}$
k_{cat}: Catalytic rate constant of the enzyme, defined in eq.3 using known values.
k_{inact}: Rate constant for the thermal inactivation of the enzyme, defined in eq.4 using known values.
K_{eq}: Equilibrium constant for the $\frac{E_{\text{act}}}{E_{\text{inact}}}$ equilibrium, calculated using known values in eq.2.
R: Gas constant, 8.314 J/mol/K.
T_{eq}: The temperature at which the $\frac{E_{\text{act}}}{E_{\text{inact}}}$ equilibrium is at $K_{eq} = 1$, one of the four parameters determined experimentally.
V_{max}: The maximum reaction rate achievable by a given quantity of an enzyme, the dependent variable of the Equilibrium Model.

Table 1: Terms used in the Equilibrium Model.

All four parameters needed to evaluate the model- ΔG^*_{cat}, $\Delta G^*_{\text{inact}}$, ΔH_{eq}, and T_{eq} - can be determined experimentally through the creation of enzyme progress curves over a variety of temperatures. The process for doing this entails collecting data on the reaction rate of the catalase-catalyzed reaction as a function of time for at least 10 temperatures and is outlined in detail in the Procedures section. Once this data is collected, it can be fit to the equation in fig.5 via Matlab to generate the above four parameters, which can in turn be used to plot the reaction rate simultaneously against temperature and time.

Equipped with a methodology for determining the shape and spread of the enzymatic activity curve for variation in temperature, it is now possible to make quantitative judgments about the thermal stability of an enzyme, and we can return to the initial question: how does the immobilization of an enzyme affect its tolerance to changes in temperature?

3 Variables

Variable	Type	Values
Temperature of Enzyme Suspensions	Independent	298 K, 303 K, 308 K, 313 K, 318 K, 323 K, 328 K, 333 K, 338 K, 343 K
Type of Enzyme Suspension	Independent	Regular Catalase, Catalase Immobilized in Alginate Gel
Time	Independent	Units: Seconds
O_2 Concentration	Dependent	Units: Parts Per Thousand

Table 2: Experimental Variables.

Variable	How it is Controlled	Why it is Controlled
Concentration of H_2O_2 Solution	A 3% H_2O_2 solution will be used for all trials.	Changing substrate concentration would be a confounding variable affecting the enzyme progress curve when we seek to isolate the effect of temperature.
Concentration of Enzyme in Suspension	The same suspensions of catalase and immobilized catalase will be used for all trials.	Changing enzyme concentration would be a confounding variable affecting the enzyme progress curve when we seek to isolate the effect of temperature.
Time Test Tubes Spend in Water Bath	All test tubes will spend five minutes in the water bath at the temperature corresponding to each trial.	Failure to regulate the time the test tubes spend in the water bath would cause the temperature of the enzyme suspension to become higher or lower than expected, compromising measurements integral to the construction of the composite temperature stability curves.
Amount of Enzyme Suspension Added to the H_2O_2 Solution	10 drops of the enzyme suspension will be added to each H_2O_2 solution for each trial.	Differing amounts of enzyme suspension added would change the concentration of the enzyme over each trial, introducing a confounding variable that would affect the enzyme progress curve when we seek to isolate the effect of temperature.

Table 3: Control Variables.

4 Materials

- Vernier Computer Interface Logger
- LoggerPro
- Vernier O_2 Gas Sensor
- 1, 400 mL beaker
- 1, 250 mL Nalgene bottle
- 3.0% H_2O_2 solution
- Catalase suspension
- Suspension of Catalse immobilized in Alginate Gel
- 4,18x150 mm test tubes
- Test tube rack
- Thermometer
- 4, dropper pipettes

5 Procedure

5.1 Regular Catalase

1. Obtain and wear goggles and safety apron.

2. Connect the Oxygen Gas Sensor to the computer interface.

3. Place four test tubes in a rack and label them 0,1,2, and 3. Fill each test tube with 3 mL of 3.0% H_2O_2 solution and 3mL of water.

4. Initiate the enzyme-catalyzed reaction for the control trial.

   (a) Record the room temperature. Using a clean dropper pipette, add 5 drops of catalase suspension to test tube 0.

   (b) Begin timing with a stopwatch or clock.

   (c) Cover the opening of the test tube with a finger and gently invert the test tube two times.

   (d) Pour the contents of the test tube into a clean 250 mL Nalgene bottle.

   (e) Place the O_2 Gas Sensor into the Nalgene bottle. Gently push the sensor down onto the bottle until it stops.

   (f) When 30 seconds have passed, click *Collect* to begin data collection.

   (g) When data collection has finished, remove the O_2 gas sensor from the Nalgene bottle, rinse the bottle with water, and dry with a paper towel.

   (h) Move the data to a stored run. To do this, choose Store Latest Run from the Experiment menu.

5. Initiate the enzyme-catalyzed reaction for each of 10 temperatures: 298 K, 303 K, 308 K, 313 K, 318 K, 323 K, 328 K, 333 K, 338 K, 343 K.

   (a) Use a hot plate to heat a 400 mL beaker of water to the desired temperature. Use a thermometer to monitor progress.

   (b) Place test tubes 1,2, and 3 in the water bath. The test tubes should be in the water bath for 5 minutes before proceeding to the next step. Record the temperature of the water bath as indicated on the thermometer.

   (c) Add 10 drops of the catalase solution to test tubes 1,2, and 3. Repeat steps 4a-4h.

   (d) Perform a least squares regression on the graph of O_2 vs time to find the average reaction rate. If this differs by more than 20% betweeen test tubes 1,2, and 3, scrap the trial for the current temperature and start over.

   (e) Repeat 5a-5d for all 10 temperatures.

5.2 Immobilized Catalase

1. Obtain and wear goggles and safety apron.

2. Connect the Oxygen Gas Sensor to the computer interface.

3. Place four test tubes in a rack and label them 0,1,2, and 3. Fill each test tube with 3 mL of 3.0% H_2O_2 solution and 3 mL of water.

4. Initiate enzyme-catalyzed reactions for control trial

   (a) Repeat steps 4a-h from section 5.1 using suspension of catalase immobilized in alginate gel.

5. Initiate enzyme-catalyzed reactions for each of 10 temperatures.

   (a) Repeat steps 5a-e from section 5.1 using suspension of catalase immobilized in alginate gel [with Vernier,].

6 Safety and Ethical Concerns

- The researcher will wear an apron and goggles, since high temperatures are involved in the lab.
- The researcher will take proper precautions when using the hot plate, including the use of hot hands to move test tubes and the maintenance of a tidy workspace free of debris.
- In the case of damage to a glass test tube, the shards will be properly disposed of with a broom and dustpan into the broken glass bin.

7 Raw Data and Progress Curve Generation

	298 K	303 K	308 K	313 K	318 K	323 K	328 K	333 K	338 K	343 K
0 s										
500 s										
1000 s										
1500 s										
2000 s										
2500 s										
3000 s										
3500 s										

Table 4: The average O2 concentration over each triplicate set of trials for each temperature at each time in the non-immobilized catalase suspension.

	298 K	303 K	308 K	313 K	318 K	323 K	328 K	333 K	338 K	343 K
0 s										
500 s										
1000 s										
1500 s										
2000 s										
2500 s										
3000 s										
3500 s										

Table 5: The average O2 concentration over each triplicate set of trials for each temperature at each time in the immobilized catalase suspension.

Graphing these results in a scatter-plot shows the rough trends that will be fitted more cleanly in the next section. Each point represents the data point from the middle trial, with error bars showing the range between the highest trial and lowest trial. Non-Immobilized catalase and Immobilized catalase are shown side by side in fig.3.

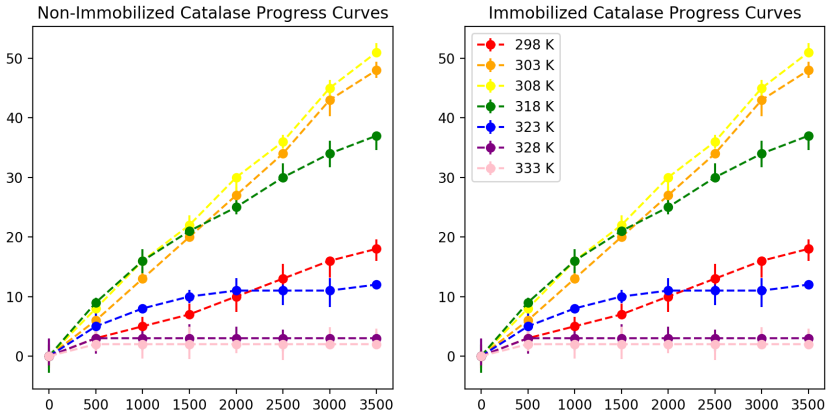

Figure 3: Raw Data for the formation of O_2 (μmols) by catalase over time at each of 10 temperatures (K).

8 Applying the Equilibrium Model

With all the necessary raw data, the Equilibrium Model fitting MatLab code provided by [Daniel, 2010] can be used to fit the data for each temperature to progress curve functions. Once the progress curves have been calculated, the four variable parameters of the Equilibrium Model can be determined by fitting the progress curve data to eq.5 using MatLab. Once estimates for each of these four parameters have been made, a three-dimensional plot or rate of product formation, temperature, and time can be generated (again using MatLab), as shown in figs. 4 and 5. Also shown in figs. 4 and 5 are the three-dimensional plot of the residuals from the fit and the thermal stability curve with those residuals mapped onto it.

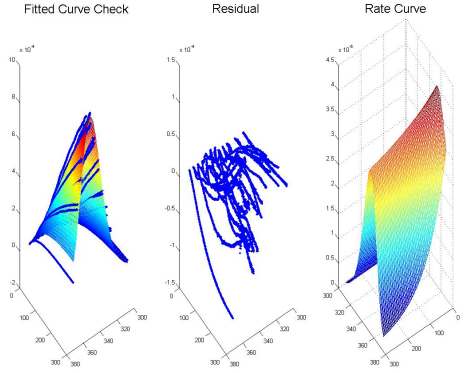

Figure 4: From left to right: The thermal stability curve with superimposed residual plot, the plot of residuals from the EM fit, and the three-dimensional thermal stability curve, calculated using the progress curves for non-immobilized catalase.

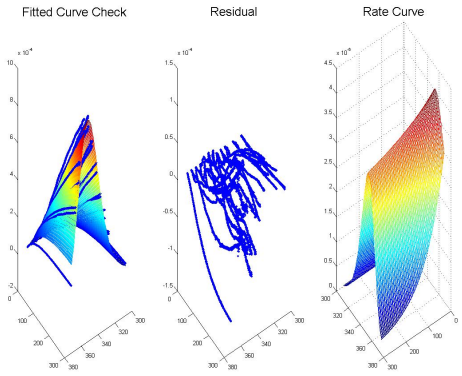

Figure 5: From left to right: The thermal stability curve with superimposed residual plot, the plot of residuals from the EM fit, and the three-dimensional thermal stability curve, calculated using the progress curves for immobilized catalase.

9 Assessing Error in Fit

The exponential nature of the Equilibrium Model (eq.5) effectively means that small inaccuracies in the data can lead to big errors in the fitting of the model to the enzyme progress curves. To attempt to evaluate the variance of the four variable parameters determined experimentally, a Monte Carlo simulation can be performed via MatLab. Through random generation of data points from a normal (Gaussian) distribution, the Monte Carlo method allows the researcher to approximate the spread of a variable in question using hundreds of thousands of iterations rather than just the few data points that have been experimentally gathered. The distributions of the four variable parameters for the non-immobilized catalase are shown in fig. 6, and those for the immobilized catalase are shown in fig. 7. As is shown in by the scale on the x axes of these graphs, each experimentally determined parameter for the Equilibrium Model has a very small standard deviation, which is a good indicator of the model's accuracy of fit. The standard deviation found through Monte Carlo iterations is then used by the MatLab software [**?**] to create a "higher resolution" thermal stability curve fit.

Additionally, the residuals from the final fit of the Equilibrium Model can be plotted. If the Equilibrium Model fits the experimental data well, there should be no apparent pattern in the distribution of the residuals. This lack of pattern is illustrated in the residual plot for the non-immobilized catalase (fig. 8) and for the immobilized catalase (fig. 9), thus showing that in this case, the data collected was precise enough to ensure a fairly accurate fit of the Equilibrium Model.

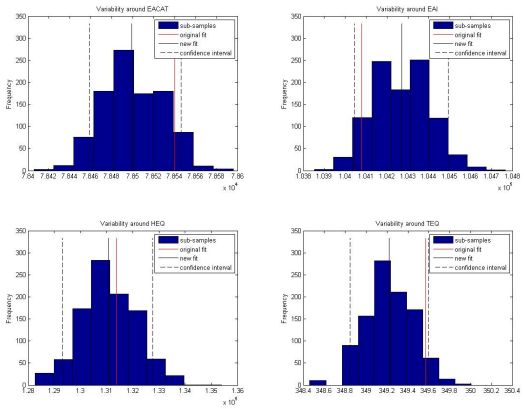

Figure 6: The distribution of ΔG^*_{cat} (EACAT), $\Delta G^*_{\text{inact}}$ (EAI), ΔH_{eq} (HEQ), and T_{eq} (TEQ) for non-immobilized catalase, as determined using the Monte Carlo method.

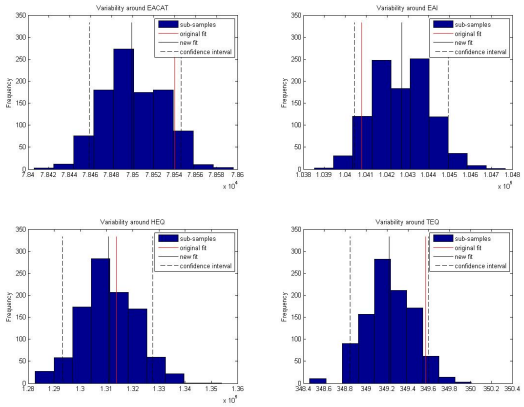

Figure 7: The distribution of ΔG^*_{cat} (EACAT), $\Delta G^*_{\text{inact}}$ (EAI), ΔH_{eq} (HEQ), and T_{eq} (TEQ) for immobilized catalase, as determined using the Monte Carlo method.

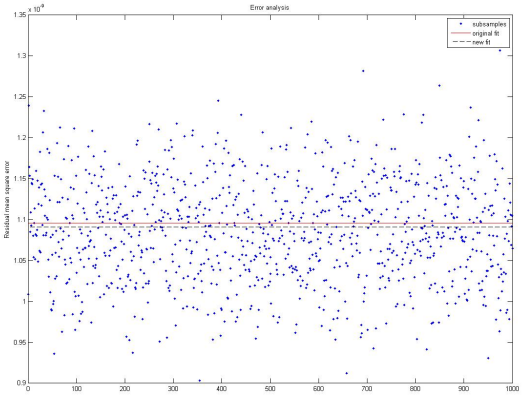

Figure 8: The residual plot from the final Equilibrium Model fit for non-immobilized catalase.

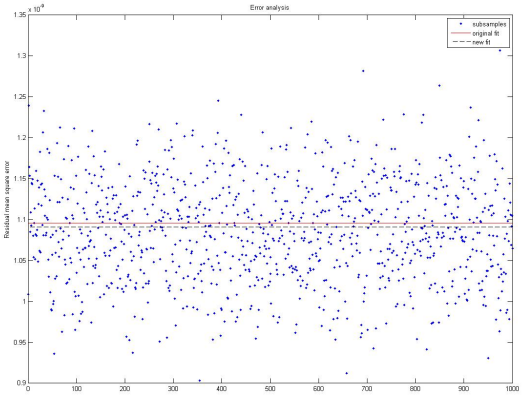

Figure 9: The residual plot from the final Equilibrium Model fit for immobilized catalase.

References

[Daniel, 2010] Daniel, R. M. (2010). A new understanding of how temperature affects the catalytic activity of enzymes. *Trends in Biochemical Sciences*, 35(10):584–591.

[Peterson, 2007] Peterson, M. E. (2007). The dependence of enzyme activity on temperature: determination and validation of parameters. *Biochem J.*, 402(2):331–337.

[with Vernier,] with Vernier, A. B. Enzyme action: Testing catalase activity (method 1- o2 gas sensor.